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(54) Title: ASSAY FOR MONITORING THE PROGRESS OF CHRONIC MYELOGENOUS LEUKEMIA

(57) Abstract

An assay for monitoring the progress of chronic myelogenous leukemia (CML) which makes possible the optimalization of the time of bone marrow transplantation. The assay is based on determination of *de novo* methylation of DNA samples, which enables the recognition of the onset of blast lysis. There is determined the *de novo* methylation of part of the *bcr/abl* gene and the degree of such methylation.

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Description

Assay for monitoring the progress of chronic myelogenous leukemia

FIELD OF THE INVENTION:

An assay for monitoring the progress of chronic myelogenous leukemia (CML). Monitoring the development of the disease makes possible to optimize the time for bone marrow transplantation. The assay is based on the determination of de novo CpG methylation, which is indicative of the progress of the disease.

BACKGROUND OF THE INVENTION:

Chronic myelogenous leukemia (CML) is a fatal clonal stem-cell disorder which accounts for about 25 percent of all leukemia cases, with an annual incidence of 1 per 100,000. It results from a reciprocal translocation, t(9;22), cytogenetically detected by the presence of the Philadelphia chromosome (Ph'). At the molecular level, the protooncogene abl located on chromosome 9 is fused to the bcr gene on chromosome 22, resulting in the formation of the bcr/abl hybrid gene. The latter, which is under the transcriptional control of the bcr promoter, gives rise to an 8 kb mRNA which is translated into a 210 kDa fusion protein

(Fig. 1A). This aberrant protein exhibits enhanced tyrosine kinase activity, and its role in the pathogensis of CML has been demonstrated by the induction of a CML-like syndrom following the introduction of bcr/abl constructs into mouse bone marrow cells.

Clinically, CML is characterized by a triphasic course, exemplifying its malignant evolution. The main finding in the initial chronic phase, which often develops insidiously, is an increased pool of committed myeloid progenitor cells whose differentiation remains uncompromised. After a few weeks to several years (median duration, 42 months), the disease enters the "acceleration" phase, later progressing to the acute phase. During these two phases, a myeloid or lymphoid progenitor, a descendent of the originally affected stem cell, loses its capacity for terminal differentiation. Sequential transformation from the chronic to the accelerated phase and blastic crisis appears to occur at random. Since the onset of the disease cannot be predicted on an individual basis, and there is no evidence that conventional antileukemic therapy at the chronic stage alters the risk of transformation, survival is determined mainly by the intrinsic biology of the disease.

In the past decade, there has been clinching evidence that many patients in the chronic phase of the disease can be

cured by bone marrow transplantation (BMT). The question is not whether to perform a marrow transplant but the optimal timing. The related mortality within the first two years of BMT is about 25% and the morbidity rate, including severe Graft Versus Host Disease (GVH-D), is even higher. Therefore, if one could accurately define a cohort of patients likely to remain in the chronic phase for several years, it would be reasonable to delay the procedure as long as possible. Such a definition cannot be based on either clinical presentation or on the results of any currently used laboratory test. It is, however, conceivable that the progression of the disease is the consequence of genetic alterations which must accumulate prior to changes in hematopoietic stem cell behavior. A great deal of effort has been invested in identifying these genetic alterations and, indeed, certain mutations and chromosomal aberrations are found to be associated with the blastic transformation. Nevertheless, there is thus far no distinct molecular finding commonly related to tumor progression in CML which precedes and can therefore predict the blastic transformation.

SUMMARY OF THE INVENTION:

The invention is based on the finding that part of the bcr/abl gene undergoes de novo methylation during the course of CML. Samples of DNA which are free of methylation at the time of initial diagnosis, invariably become methylated before the blast crisis. The degree of methylation can be monitored and indicates the progress and stage of the disease. This finding, and the assay based on it, is indicative of the progress of the disease and can be used to predict the onset of the blast crisis, and this can be used for the optimal timing of bone marrow transplantation.

The present invention provides an accurate assay for the detection and evaluation of the progress of CML.

According to the invention there are provided means for carrying out the assay and for the evaluation of the results. A novel kit is also provided for carrying out such an assay.

An assay is provided for determining the state of progress of chronic myelogenous leukemia (CML) based on determining de novo methylation of part of the bcr/abl gene and the degree of such methylation, where DNA segments are first subjected to the action of certain site-methylation sensitive restriction endonucleases and then to PCR analysis where the failure of cutting with certain emdonucleases is indicative of partial methylation while failure of cutting with other endonucleases indicates full methylation. Amongst others there may be used site-methylation sensitive restriction enzymes such as HpaII, KspI (SacII), HincII BssH-II and Not-1 and the site-methylation insensitive enzyme Msp1 as a control.

A preferred embodiment comprises preparing high molecular weight DNA of the patient's blood cells, digesting multiple samples of DNA each with one of the enzymes defined above, running the PCR reaction using the primers Ia3 together with either primer CG20 or CA20 for each restriction product; isolating the PCR products of each reaction, spotting an aliquot of the material on a membrane disk, hybridizing each disk with a biotinilated Ia5 probe, incubating with avidin peroxidase and developing the reaction with a chemiluminescence kit.

Preferably the PCR product of each reaction is isolated by the use of a spin column, using pass-through material for the next step.

The PCR product can be analysed by gel-chromatography and ethidium bromide staining or by Southern blot with Ia5 as a probe. Other primers for the PCR reaction can be chosen according to the abl Ia promoter sequence

5'-TCTCCGGGCCCTTTGTTAACAGGCGCGTCCCGGCCAGGCGGAGACGGCCGGCCATGG...
CGGGGGGGCGCGCGGGCCGAGCCGGGCCTGAGCCGGGCCCGCGGACCGAGCTGG
GAGAGGCTCCGGCCCCCGACGTCGTGGCGCGGGAAAATGTTGGAGATCTGCCTGA
AGCTGGTGGGCTGCAAATCCAAGAAGGGGCTGTCCTCGTCCTCCAGCTGTTATCTGG-3'

If one of the site methylation sensitive enzymes used does not cut the DNA, partial methylation is present. If none of the site methylation sensitive enzymes cuts, this is indicative of full methylation. The activity of the specific enzymes and the results are fully illustrated in the following. The effect of various enzymes is indicative of the degree of methylation.

An example of a kit for carrying out the assay of the present invention comprises in combination the following:

A) DNA primers:

Ia3 5' -GGAAGCTTGATAACAGCTGGAGGAC-3'

CG20 5' -CGGGGGGGCGCGCGGGCCGA-3'

ca20 5' -CTCCGGGCCCTTTGTTAACA-3'

Ia5 5' -GGTCTAGACAAAATGTTGGAGATCTG-3'

B) Restriction enzymes Hpa II, Msp I, Kspl (SacII), BssH II, Hinc II and Not-1.

C) PCR reaction mix

D) PCR product isolation - spin columns

E) Membrane discs

F) Hybridization mix

G) Avidin Peroxidase

H) Instruction Manual

The basic steps of the assay, based on the use of such a kit, or on components of this types or their equivalents is as follows:

a) Prepare high molecular genomic DNA according to Ausubel et al., Current Protocols in Mol. Biology Wiley Interscience (1993).

b) Digest a number of identical samples of DNA with each enzyme.

c) Using the primers 1a5 and either CG20 or CA20 run PCR reaction for each restriction product.

d) Isolate PCR product of each reaction with the spin column.

e) Spot an aliquot of the pass-through material from each spin column onto a membrane disc.

f) Hybridize each disc with biotinilated Ia5 primer.

g) Wash and incubate with Avidin peroxidase.

h) Wash and develop the reaction with a chemiluminescence kit.

Clinical diagnosis of CML is usually made in the chronic phase of the disease, its onset, whether several days or years ago, remaining obscure. With time, the affected stem cell is prone to secondary, cumulative alterations which eventually result in blastic crisis. At that phase, bone marrow transplantation, the only potentially curative procedure in CML, is no longer effective. Determination of the rate of progress is, therefore, of prime importance in the management of the disease.

The assay of the invention provides evidence for a distinct and common molecular event in CML, the de novo methylation of the CpG island associated with the abl Pa promoter. In CML, the process of the Pa CpG methylation is of a progressive nature, ultimately apparent in every patient who has reached the accelerated and blastic phases of the disease. The course of the disease may be monitored by the extent of CpG methylation. While the clinical presentation at the chronic stage may be misleading, partial methylation at the time of diagnosis in the chronic phase indicates a more advanced form of the disease. A molecular marker for CML progression, such as the Pa methylation pattern, point to the immediate necessity for BMT.

The following description sets out in detail how the assay of the invention can be performed. This description is by way of illustration only, and it is clear that various changes and modifications in the nature of components and sequence of certain steps cna be resorted to without departing from the scope and spirit of the invention.

MATERIALS AND METHODS

1. Cell lines

The Ph'-positive CML cell lines: BV173, KBM5, EM2, and K562 (20); a Ph'-positive acute lymphoblastic leukemia (ALL) cell line, ALL1; TRF, Daudi, Raji and HL60 were grown in RPMI-1640. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% fetal calf serum, 2mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37°C and 5% CO_2.

2. Patients' Samples

Patients whose DNA samples were analyzed by PCR, were diagnosed in either the Hematology Department at Hadassah Medical Center or at the M.D. Anderson Cancer Center. CML diagnosis was based on clinical evaluation and laboratory tests. These included WBC and platelet counts, spleen size, hemoglobin level, chromosomal analysis and RT-PCR analysis of *bcr/abl* mRNA. Disease stage was determined according to the criteria of the Bone Marrow Transplant Registry for Classifying the Phases of CML (22).

3. Northern blot analysis

RNA was extracted from the cells by the $LiCl_2$-urea method (23). Poly adenylated (Poly A^+) RNA was obtained by oligo (dT)-cellulose chromatography. A 5 μg quantity of Poly A^+ RNA was electrophoresed through 0.7% formaldehyde agarose gel and blotted on a nitrocellulose filter. Filters were pretreated for 2 hr at 65°C in a solution of 1M NaCl, 1% SDS with 150 μg/ml salmon sperm DNA and hybridized with a ^{32}P-random primed purified cDNA fragment, P18. Hybridization was carried out at 65°C for 18 hr in a solution containing 10%

dextran, 1M NaCl, 1% SDS and 200 μg/ml salmon sperm DNA. The filters were briefly washed in 2xSSC and then subjected to four additional washings (20 min, 65°C) in a solution of 0.1 x SSPE and 1% SDS.

4. RNase protection analysis

A 0.64 kb NotI-BamHI fragment isolated from the *abl* Ia genomic region was inserted into a pGEM-3Zf(+), and ^{32}P-labeled antisense RNA was synthesized *in vitro*. The probe was annealed to 3-5μg of poly A$^+$ RNA from various cell lines. The annealed products were subjected to RNase treatment as previously described (24), and the protected fragments were separated on urea-acrylamide gel.

5. Southern blot analysis

Isolation of genomic DNA from cell lines and bone marrow was as described (24). A 10-20μg quantity of genomic DNA was digested with either HindIII or a combination of HindIII and SacII or HindIII and NotI restriction enzymes. After electrophoresis through a 1% agarose gel, the DNA was blotted on a nylon membrane (Hybond N, Amersham) under alkaline conditions for 7 hr. The membranes were hybridized with a random primed ^{32}P genomic HincII-PvuII 240 bp Ia probe. Hybridization was carried out under the same conditions as in the Northern blot analysis.

6. DNA sequencing

A 240 bp HincII-PvuII fragment containing the Ia exon and its 5' flanking region was cloned into M13 and sequenced using the dideoxynucleotide chain-termination method.

7. PCR analysis

Cell line and bone marrow genomic DNA's were extracted as described. A 400 ng quantity of genomic DNA was digested with 5 units of one of the following enzymes: BamHII, HpaII, MspI, KspI (SacII), BglII (Boehringer Mannheim) for 6 hours in 20μl aliquots. To ensure complete cleavage, an additional 5 units of the enzyme were added and digestion was allowed to proceed for an additional 16 hours. PCR amplification of the reaction products was performed as follows: 10 μl containing 200 ng of the cleaved genomic DNA were added to 50μl of PCR mixture containing: 500μM dNTP's, 67mM Tris Hcl, ph 8.8, 3mM $MgCl_2$, 16.6 mM $(NH_4)_2$ SO_4, 10mM ß-mercaptoethanol, 10% DMSO and 10 pmoles of Ia3 primer (5'-GGAAGCTTGATAACAGCTGGAGGAC-3'). The reaction mix was preheated for 10 min at 94°C and then 10 pmoles of CG20 primer (5'-CGGGGGGGCGCGCGGGCCGA-3') and 2.5 units of Taq DNA polymerase (Boehringer Mannheim) were added at 85°C. This was followed by 28 cycles of 1 min at 94°C, 1 min at 59°C and 1 min at 72°C, terminated by 10 min at 72°C in a Perkin Elmer Cetus thermocycler. A 10μl aliquot of the amplified product was separated by electrophoresis through 4% Nusieve agarose/0.5% agarose gel, transferred to a nylon membrane (Hybond N, Amersham), as described in the Southern blot analysis and prehybridized for 2 hours at 42°C. The hybridization solution contained 20% formamide, 5x Denhardt's solution, 6xSSC, 0.1% SDS, 0.05% PPi and 25 μg/ml tRNA. The Ia5 internal oligomer (5'-GGTCTAGACAAAATGTTGGAGATCTG-3') was ^{32}P end labeled and used as a probe. After 2 hr hybridization at 42°C, the membrane was washed at room temperature for 10 min with 6xSSC and exposed to X-ray film.

Expression of the normal *abl* 6 kb mRNA is absent in CML cell lines lacking an intact chromosome 9.

The protooncogene *abl* is expressed in all normal human cells. The gene has two first alternative exons, Ia and Ib, which are differentially transcribed from their own promoters (fig. 1A). The proximal promoter, Pa and a distal one, Pb are 175 kb apart and direct the synthesis of two mRNA species of 6 and 7 kb, respectively (25). In approximately 90% of Ph' translocations, the proximal promoter (Pa) is nested within the *bcr/abl* transcriptional unit (Fig. 1A) The position of Pa, respective to the *bcr* promoter, is often analogous to that in the native gene, where it is nested within the Pb transcriptional unit, 27). In normal cells, both *abl* promoters are active in gene expression; the activity of the Pa downstream promoter appears to be unaffected by the upstream Pb promoter. To date, however, the transcriptional activity of Pa within the *bcr/abl* gene has not been investigated.

Certain CML cell lines established from CML patients in blast crisis carry multiple Ph' chromosomes, but have no intact chromosome 9. In our experiments, the only mRNA species found to hybridize with the *abl* probe was the 8 kb *bcr/abl* transcript common to all CML cells (Fig. 1B). The identity of the 8 kb band as the *bcr/abl* transcript was confirmed using a *bcr* probe (data not shown). In K562, a CML cell line with multiple Ph' chromosomes in addition to an intact chromosome 9, the normal *abl* 6 and 7 kb mRNAs were observed together with the 8 kb *bcr/abl* transcript (Fig. 1B).

RNase protection assay confirmed the observation that the BV173, EM2 and KBM5 cell lines did not express the 6 kb mRNA initiated from the main Pa transcriptional start point, whereas it was expressed in the K562 cell line (Fig. 1C). Hence, it appears that the translocation event compromises Pa transcription from the Ph' chromosome but spares the transcriptional activity of *abl* in the unaffected chromosome.

.The Pa promoter is nested within a CpG island which is methylated in CML cell lines.

The loss of Pa transcriptional activity in the various CML cell lines led us to study the Pa promoter region. The genomic DNA upstream of the Pa exon is composed of 85% CG (Fig. 2B). In vertebrate genomes, the dinucleotide sequence CpG is relatively rare. It is often clustered in a discrete region of about 1 kb, mostly surrounding promoters, a sequence known as CpG island. While the observed to expected (O/E) ratio of CpG throughout most of the genome is less than 0.25 and in the Ia exon was 0.157, the figure for the Pa promoter region was 1.06, nearly the expected value. Furthermore, restriction enzyme analysis showed multiple restriction sites for certain rare cutting enzymes such as NotI, SacII, BssHII, and SmaI (Fig. 2A), a feature typical of CpG islands

According to the RNase protection assay and the sequence characteristics of the Pa region, the main transcriptional start point of the Pa promoter was localized within the CpG island (Fig. 2A).

CpG islands are generally methylation free, whether associated with transcriptionally active or inactive genes. The rare exceptions where the

CpG islands are methylated, including X-chromosome inactivation and imprinted genes, involve genes that are initially active and undergo repression during embryonic development. As it seemed likely that the activity of the Pa promoter on the Ph' chromosome could also undergo such a process, becoming repressed following the translocation event, we examined the methylation status of the CpG island.

To probe for methylation in the CpG island, advantage was taken of several site-methylation sensitive restriction enzymes. When genomic DNA is digested with HindIII, which cuts outside the CpG island, a 10.4 kb band is produced (Fig. 3A). Digestion with HindIII and NotI or SacII (site-methylation sensitive enzymes) results in a 2.5 or a 2.3 kb Ia *abl* hybridizing band, respectively, in the unmethylated state (Fig. 3A). Double enzymatic digestion of DNA from the non-CML cell line HL60, produced the small hybridizing fragments (Fig. 3B). In CML cell lines lacking chromosome 9, such as BV173, KBM5, and EM2, only the large HindIII 10.4 kb fragment appeared. Double digestion of genomic DNA from K562, which has the normal chromosome 9 and several Ph' chromosomes, generated both large and small hybridizing fragments (Fig. 3B). The intensity of the large hybridizing fragment was several fold stronger than that of any of the small fragments. Since four copies of the Ph' chromosome are present in K562 the more pronounced hybridizing band is most likely derived from the Ph' chromosome. In K562 and BV173, the Pa region is part of the Ph' chromosome, since the translocation had been documented upstream of this region. Apparently, methylation of the Pa CpG island, as judged by the observed 10.4 kb fragment, was restricted to the Pa promoter of the Ph' chromosome, while the CpG island in the normal *abl* allele was hypomethylated as expected.

The methylation status of the Pa promoter was further studied using PCR analysis. Genomic DNA was cut with one of the following restriction enzymes: BamHI, HpaII, MspI, SacII, or BglII and the digested fragments were amplified, using oligonucleotides CG20 and Ia3 (Fig. 2B). Following BamHI digestion, DNA amplifiction resulted in a 103 bp fragment encompassing a cluster of three HpaII sites and a single SacII site. This fragment hybridized with the radiolabeled internal oligonucleotide Ia5 (Fig. 2B). Complete methylation of the three HpaII sites, or of the SacII site was essential for generation of the PCR product. Following digestion with these enzymes, the CML lines, but not the control cells, generated the 103 bp fragment. Hence all these sites appear to be methylated in the CML lines (Fig. 3C). No PCR product was detected when the DNA was digested with enzymes insensitive to methylation at CpG, such as MspI and BglII (Fig. 3C). For a positive internal control, each DNA sample was amplified subsequent to HpaII digestion with the Ia5 and Ia3 primers (Fig. 2B) and yielded the expected 78 bp fragment (data not shown).

Progressive Pa methylation in CML patients

Methylation of a CpG island, while rare *in vivo*, is often observed in cells grown in culture for many generations. Therefore, findings characteristic of CML cells are not necessarily indicative of the native status of the CpG island. In any event, the CML cell lines are derived from late stage (blast crisis) of CML and cannot provide information on the methylation state in the early phase of the disease. Therefore there were tested DNA samples from 18 CML patients in various phases of the disease and 4 non-leukemic subjects (Table 1). Three methylation patterns were observed: Pattern A - no methylation at HpaII or

SacII. Pattern B - partial methylation, the SacII site is methylated and at least one HpaII site is non-methylated. Pattern C - complete methylation of all HpaII and SacII sites.

10 of the 14 chronic phase samples exhibited pattern A, with the remainder displaying partial methylation (pattern B) (Table 1). In contrast, of the 3 samples from patients at the accelerated phase, 2 exhibited pattern B while the third showed complete methylation of the Pa CpG island (Fig. 4A and Table 1). Of the 11 blast crisis samples, 10 were classified as C and only 1 exhibited partial methylation (Table 1).

In following the course of methylation, DNA samples from both the chronic phase and the blast crisis of 5 patients were studied (Fig. 4B). In the blast crisis all 5 patients showed evidence of progressive methylation at the Pa promoter region. The 4 patients who had shown no indications of methylation in the chronic phase, displayed either pattern C (3 patients) or pattern B (1 patient) at blast crisis. The fifth patient, who exhibited pattern B in the chronic phase, progressed to pattern C.

Discussion

Clinical diagnosis of CML is usually made in the chronic phase of the disease, its onset, whether several days or years ago, remaining obscure. With time, the affected stem cell is prone to secondary, cumulative alterations which eventually result in blastic crisis. At that phase, bone marrow transplantation, the only potentially curative procedure in CML, is no longer

effective. Determination of the rate of progress is, therefore, of prime importance in the management of the disease.

The terminal stage of the disease is associated with multiple chromosomal aberrations and other genetic alterations. Several chromosomal aberrations are found in blast crisis cells, particularly amplification of the Ph' chromosome, isochromy 17q and trisomy 8. Activating point mutations in Ras family genes and rearrangements in p53 gene occur in only a small proportion of CML blast crisis patients. Thus, with the exception of the Ph' duplication, the variety of genetic changes occurring in terminal CML are neither unique nor is there evidence to suggest that any one of them leads to the eruption of the blast crisis. Here, we provide evidence for a distinct and common molecular event in CML, the *de novo* methylation of the CpG island associated with the *abl* Pa promoter. Changes in DNA methylation have been noted in different types of cancer, including CML, and are sometimes apparent only in advanced phases. Some of these events are sporadic; the more common ones may be implicated in tumorigenesis but need not specifically reflect tumor progression. In CML, the process of the Pa CpG methylation is of a progressive nature, ultimately apparent in every patient who has reached the accelerated and blastic phases of the disease. This pattern suggests that the course of the disease may be documented by the extent of CpG methylation. While the clinical presentation at the chronic stage may be misleading, partial methylation at the time of diagnosis in the chronic phase might indicate a more advanced form of the disease. A molecular marker for CML progression, such as the Pa methylation pattern, could point to the immediate necessity for BMT.

Aside from generation of the *bcr/abl* product, another consequence of the Ph' translocation is the disruption of one *bcr* and one *abl* allele in the affected stem cell. Were both alleles of each gene expressed prior to the translocation, disruption of the *abl* gene would result in 50% loss of the relevant mRNA. However, the situation is more complex, since in most cases the translocation leaves one transcriptional *abl* unit (Pa) within the Ph' chromosome and only the Pb unit is compromised (Fig. 1A). Surprisingly enough, we have found that in CML cell lines lacking the normal *abl* alleles and retaining only the Ph' alleles, there is no evidence of either Pb or Pa *abl* transcription.

Assuming the transcription defect is specific to the Ph' allele, it is not known whether the lack of Pa transcription is an immediate or a late consequence of the translocation. Immediate inactivation of the Pa transcription unit following translocation would result in an abrupt decline in *abl* transcription and could therefore be correlated with the onset of the disease. Later blockage of *abl* transcription might have an impact on disease progression. Since overexpression of *abl* retards cell cycle progression *abl* may act as a growth suppressor gene or an antioncogene. It is conceivable that even a modest decline in normal *abl* expression could affect the ratio of oncogene (*bcr/abl*) to antioncogene (*abl*) expression and further promote tumorigenesis. Indeed, the ratio of the transforming N-*ras* mutant to the N-*ras* protooncogene has been found to affect tumorigenesis in neuroblastoma cell lines.

A recent observation concerning the origin of the t(9;22) translocation, hints that genomic imprinting may underlie the pathogenesis of CML. The translocation always occurs between a maternal *bcr* allele and a paternal *abl*

allele, but it is not known whether the choice of partner alleles is made prior to the translocation or if it stems from the selective advantage enjoyed by certain random combinations. Genomic imprinting could play a role in selecting the partner alleles for translocation and would account for the compromized *abl* Pa transcription observed in CML cells. For example, the translocation may involve selection of an imprinted, inactive *abl* allele, and an active *bcr* allele. Alternatively, following translocation, an imprinted *bcr* allele could impose the inactivation of an active *abl* allele, analogous to the proposed relationship between the active Igf-2 allele and the inactive H19 allele residing 90 kb apart on a paternal chromosome. Further support for *abl* and *bcr* imprinting is provided by the differential DNA replication time of imprinted alleles. Both *bcr* and *abl* belong to a group of genes including the 4 known imprinted genes, where the two alleles do not replicate synchronously. Progressive methylation has been noted as a consequence of allele inactivation. It is apparently important in maintaining the inactivated state since methyl-transferase mutant mice fail to maintain the normal pattern of genomic imprinting. Similarly, progressive methylation at the site of the Ph' translocation may serve to repress the activity of adjacent genes, either *abl* or other as yet unidentified genes. The observed methylation, apart from being a marker of tumor progression in CML, could also contribute to the process of blastic transformation.

Fig. 1: Expression of the normal *abl* 6 kb mRNA.

A. Elements participating in the generation of the *bcr-abl* gene. The translocation occurs inside the *abl* and *bcr* genes on chromosomes 9 and 22, respectively. In 90% of the patients it is upstream of the Ia exon of the *abl* gene, mostly along the large first intron.

B. Northern blot analysis of CML and control cell lines. Poly A^+ RNA from Daudi, HeLa, K562, EM2, KBM5 and ALL1 cell lines was electrophoresed through a formaldehyde agarose gel and blotted on a nitrocellulose filter. The filter was hybridized with a ^{32}P-labeled cDNA fragment, P18, corresponding to the *abl* exons Ia, II, III. The three *abl*-related mRNAs (the 8 kb *bcr/abl* fusion transcript and the normal 6 kb and 7 kb transcripts) are indicated in the figure. The 7 kb ALL1 band corresponds to the acute lymphoblasic leukemia specific *bcr/abl* fusion transcript. Note the additional minor *bcr/abl* of 7.7 kb in KBM5.

C. RNase protection analysis. A 0.64 kb NotI-BamHI genomic *abl* Ia template including exon Ia and its 5' flanking region was used for the *in vitro* synthesis of a ^{32}P-labeled antisense RNA probe. The probe was annealed to 3-5μg of poly A^+ RNA derived from K562, BV173, KBM5, and EM2 CML cell lines; ALL1-a Ph' positive ALL cell line; Daudi, Raji, HL60, TRF and HeLa cells were used as control cell lines. Yeast tRNA (5μg) was used as a negative control. The Ia protected fragment of 150 bp, indicated by an arrow, was also hybridized with two extra probes of different sizes. The number of Ph'

chromosomes in each cell line is indicated. A ^{32}P-labeled pBR322 MspI digest was used as a size marker. Appropriate protected fragments were produced in all cases when using exon Ib as a riboprobe for RNA quality control (data not shown).

Fig. 2: Characterization of the Pa promoter.

A. Schematic organization of the *abl* gene and restriction map of the CpG island containing the Ia exon and its 5' flanking region. Boxes represent exons. Arrows represent transcriptional start sites.

B. Sequence of the Ia exon and its 5' flanking region. The arrows denote oligonucleotides length and directionality. The starting ATG marked with an arrow and the restriction sites are underlined.

Fig. 3: Methylation state of the CpG island of the Ia exon genomic region.

A. Physical map of the normal human *abl* locus. The exon is depicted as a box. Restriction fragments and their sizes are indicated.

B. Southern blot analysis.

A 20µg quantity of genomic DNA from the control cell line HL60 and CML cell lines EM2, KBM5, BV173 and 10µg of K562, were digested with HindIII (H)*HindIII and SacII (HS) or HindIII and NotI (HN). The obtained DNA's fragments were hybridized with a ^{32}P-genomic *abl* Ia probe. The number of Ph' chromosomes is indicated underneath each cell line. Lambda HindIII DNA marker sizes in kb were visualized with ethidium bromide staining.

C. Assessment of Pa promoter methylation status using PCR.
Genomic DNA's were extracted from human WBC (Buffy Coat) and various cell lines. Following digestion with the indicated restriction enzymes, the samples were amplified by PCR using the primers CG20 and Ia3 shown in Figure 2B. The amplified DNA was analyzed by Southern blotting using the internal primer Ia5 as a probe (Fig. 2B).

Fig. 4: Progressive Pa methylation in CML patients

A. The methylation state of the Pa promoter in the accelerated phase.
Genomic DNA was extracted from the bone marrow of three CML patients in the accelerated phase. Methylation of the Pa promoter was tested as described in Fig. 3C, using PCR analysis.

B. Methylation state of the Pa promoter in chronic and blast crisis phases.
Genomic DNA was extracted from cells of five patients (A-E) in the chronic phase as well as in blast crisis. The methylation state of the Pa promoter was tested as described in Fig. 3C, using PCR analysis.

Table 1: Methylation state of the Pa promoter in the course of CML.
Patients in different phases of CML displaying three types of methylation patterns: A = no methylation; B = partial methylation; C = complete methylation. "+" represents the appearance of an Pa specific PCR product after digestion with the indicated restriction enzyme.

TABLE 1

No. of patients

Methylation Code	Chronic Phase	Accelerated Phase	Blast Crises	Normal Controls
A	10	0	0	4
B	4	2	1	0
C	0	1	10	0

Methylation Pattern

Methylation Code	BamHI	HpaII	MspI	SacII	BglII
A	+	-	-	-	-
B	+	-	-	+	-
C	+	+	-	+	-

Assay for monitoring the effects of therapy in CML patients

Patients with CML are treated with two modalities: α interferon with or without hydroxyurea, or bone marrow transplantation. The current monitoring of treatment includes complete blood count, bone marrow examination for morphology, cytogenetics and molecular analysis of the bcr/abl mRNA. None of these methods can predict the blastic transformation potential of the residual Philadelphia positive cells following therapy.

Our preliminary data indicates that interferon treated patients who respond well to therapy reversed their progressive methylation status while their cytogenetic picture did not change.

Two patients who improved their methylation status upon therapy, reverted to progressive methylation after a few months of continued therapy. This finding preceded any signs of clinical deterioration by several months at which time the patients entered the blast crisis phase of the disease.

We therefore propose our methylation assay as a tool for monitoring the outcome of therapy.

The methylation assay can be further used to evaluate novel therapeutic modalities (e.g. new differentiating agents) in ex-vivo systems, for example, SCID mice transplanted with human bone marrow.

CLAIMS

1. An assay for determining the state of progress of chronic myelogenous leukemia (CML) which comprises determining the de novo methylation of part of the bcr/abl gene and the degree of such methylation.

2. An assay according to claim 1, where DNA segments are first subjected to the action of site-methylation sensitive restriction endonucleases and then to PCR analysis where the failure of cutting with certain emdonucleases is indicative of partial methylation while failure of cutting with other endonucleases indicates full methylation.

3. An assay according to claim 2, where the site-methylation sensitive restriction enzymes are HpaII, KspI (SacII), HincII BssH-II and Not-1 and the site-methylation insensitive enzyme used as a control is Msp1.

4. An assay according to any of the claims 1 to 3, which comprises preparing high molecular weight DNA of the patient's blood cells, digesting multiple samples of DNA each with one of the enzymes defined above, running the PCR reaction using the primers Ia3 together with either primer CG20 or CA20 for each restriction product;

isolating the PCR products of each reaction, spotting an aliquot of the material on a membrane disk, hybridizing each disk with a biotinilated Ia5 probe, incubating with avidin peroxidase and developing the reaction with a chemiluminescence kit.

5. An assay according to claim 4, where the PCR product of each reaction is isolated by the use of a spin column, using pass-through material for the next step.

6. An assay according to claim 4, where the PCR product is analysed by gel-chromatography and ethidium bromide staining or by Southern blot with Ia5 as a probe.

7. An assay according to claims 4-6 where other primers for the PCR reaction are chosen according to the abl Ia promoter sequence

5'-TCTCCGGGCCCTTTGTTAACAGGCGCGTCCCGGCCAGGCGGAGACGGCCGGCCATGG...
CGGGGGGGCGCGCGGGCCGAGCCGGGCCTGAGCCGGGCCCGCGGACCGAGCTGG
GAGAGGCTCCGGCCCCCGACGTCGTGGCGCGGGAAAATGTTGGAGATCTGCCTGA
AGCTGGTGGGCTGCAAATCCAAGAAGGGGCTGTCCTCGTCCTCCAGCTGTTATCTGG-3'

and equivalent sequences where one or more nucletide has been changed to another.

8. An assay for determining the stage of development of CML based on the determination of de novo methylation of DNA samples, substantially as hereinbefore described.

9. A kit for carrying out an assay for determining the onset of blast lysis as claimed in any of the claims 1-6, which comprises in combination:

A) DNA primers:

Ia3 5' -GGAAGCTTGATAACAGCTGGAGGAC-3'

CG20 5' -CGGGGGGGCGCGCGGGCCGA-3'

ca20 5' -CTCCGGGCCCTTTGTTAACA-3'

Ia5 5' -GGTCTAGACAAAATGTTGGAGATCTG-3'

B) Restriction enzymes Hpa II, Msp I, Kspl (SacII), BssH II, Hinc II and Not-1.

C) PCR reaction mix

D) PCR product isolation - spin columns

E) Membrane discs

F) Hybridization mix

G) Avidin Peroxidase

H) Instruction Manual

10. Assay for determining the state of progress of CML, based on de novo methylation of part of the bcr/abl gene, substantially as herein described.

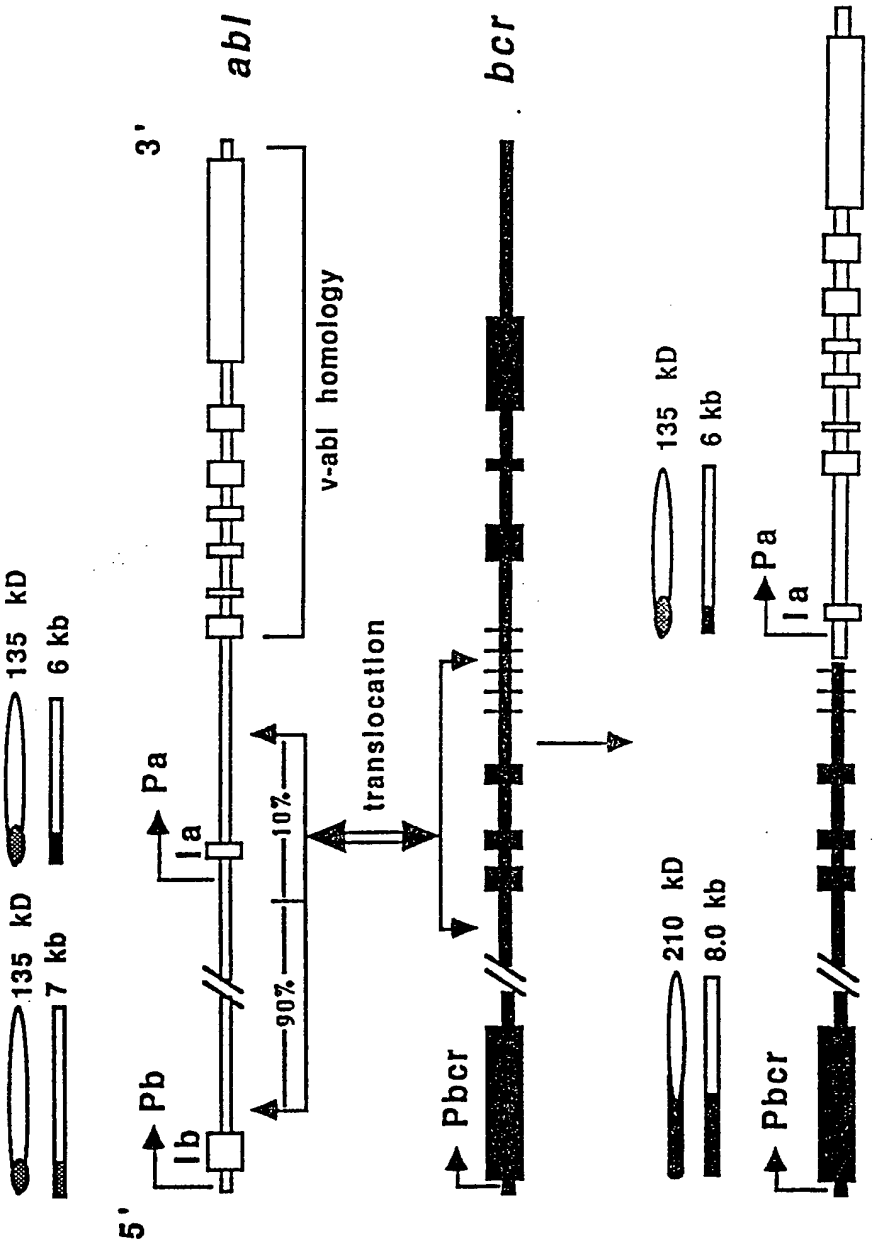
135 kD
6 kb
135 kD
7 kb
Pb
Ib
5'
3'
abl
Pa
Ia
90%
10%
v-abl homology
translocation
bcr
Pbcr
135 kD
6 kb
210 kD
8.0 kb
Pa
Ia
Pbcr

Fig. 1A

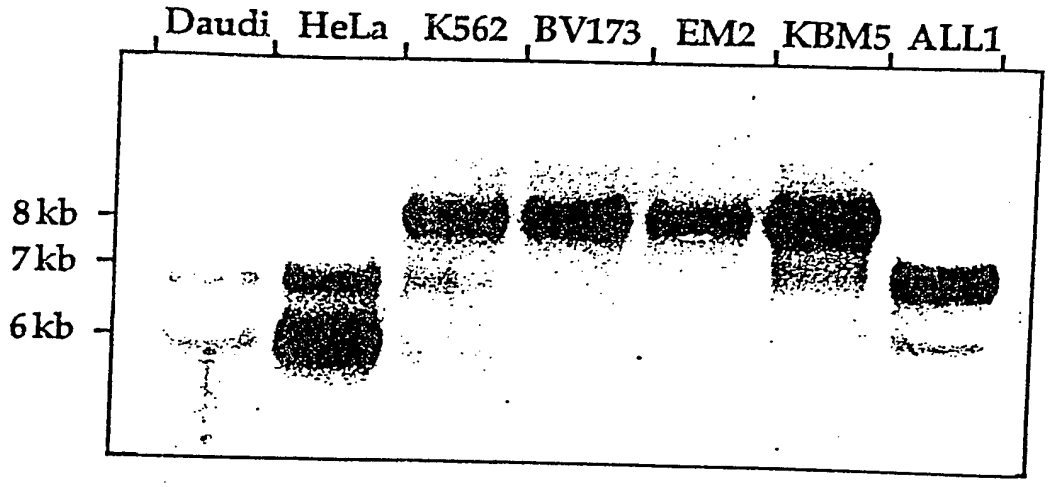

Fig. 1B

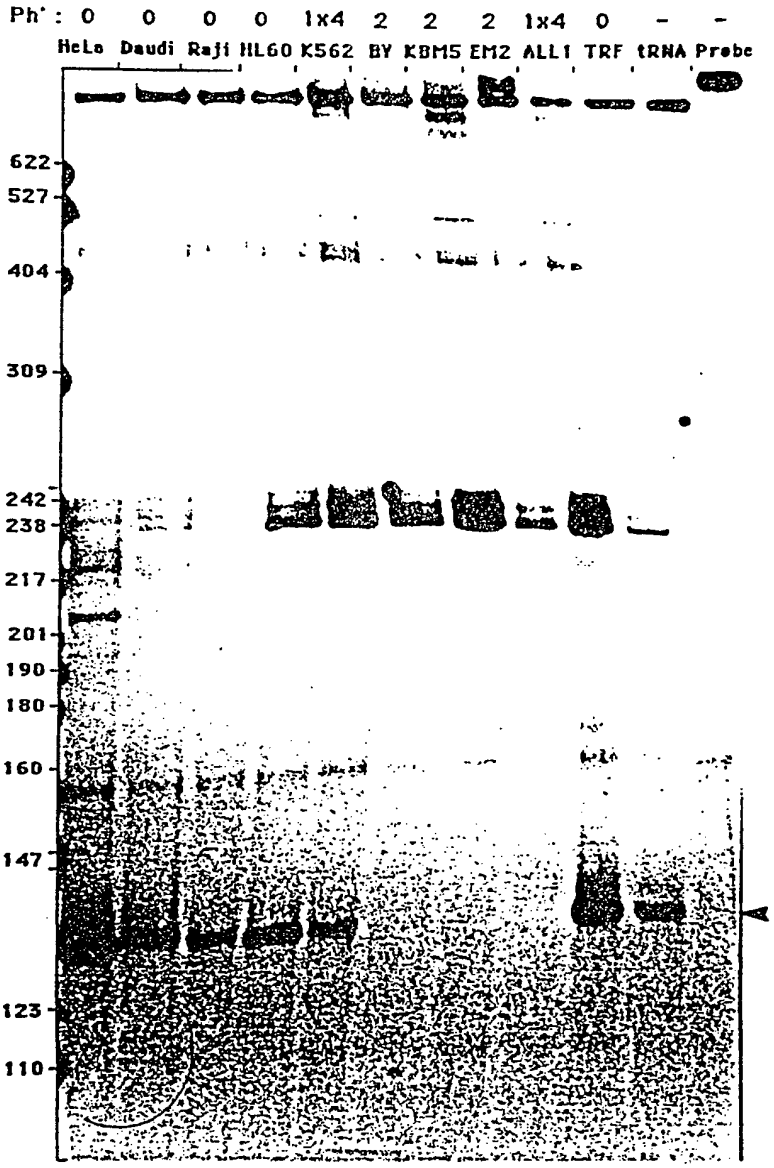
Ph+: 0 0 0 0 1x4 2 2 2 1x4 0 - -
HeLa Daudi Raji HL60 K562 BV KBM5 EM2 ALL1 TRF tRNA Probe
622
527
404
309
242
238
217
201
190
180
160
147
123
110

Fig. 1C

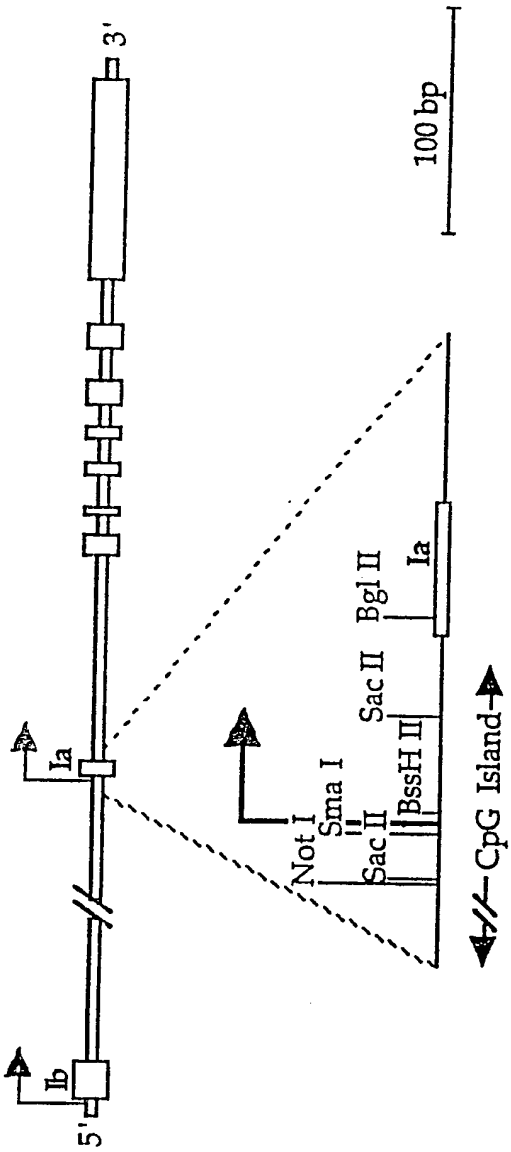
3'
100 bp
Ia
Bgl II
Sac II
BssH II
Sma I
Not I
Sac II
CpG Island
Ia
Ib
5'

Fig. 2A

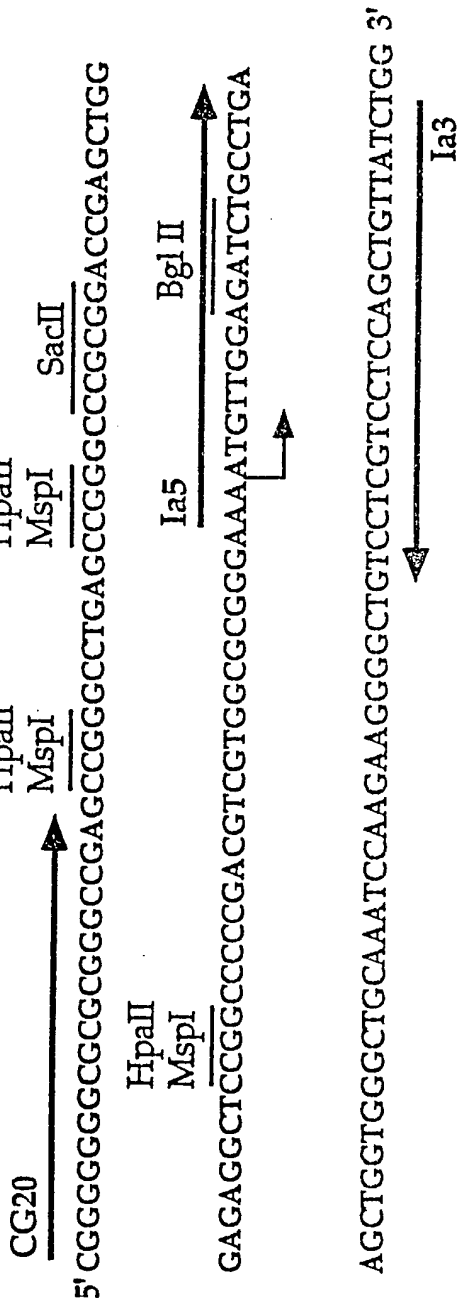
CG20
HpaII
MspI
HpaII
MspI
SacII
5' CGGGGGGGCGCGGGCCGAGCCGGGGCCTGAGCCGGGCCCGCGGACCGAGCTGG
HpaII
MspI
Ia5
Bgl II
GAGAGGCTCCGGCCCCCGACGTCGTGGCGCGGGAAAATGTTGGAGATCTGCCTGA
AGCTGGTGGGCTGCAAATCCAAGAAGGGGCTGTCCTCGTCCTCCAGCTGTTATCTGG 3'
Ia3

Fig. 2B

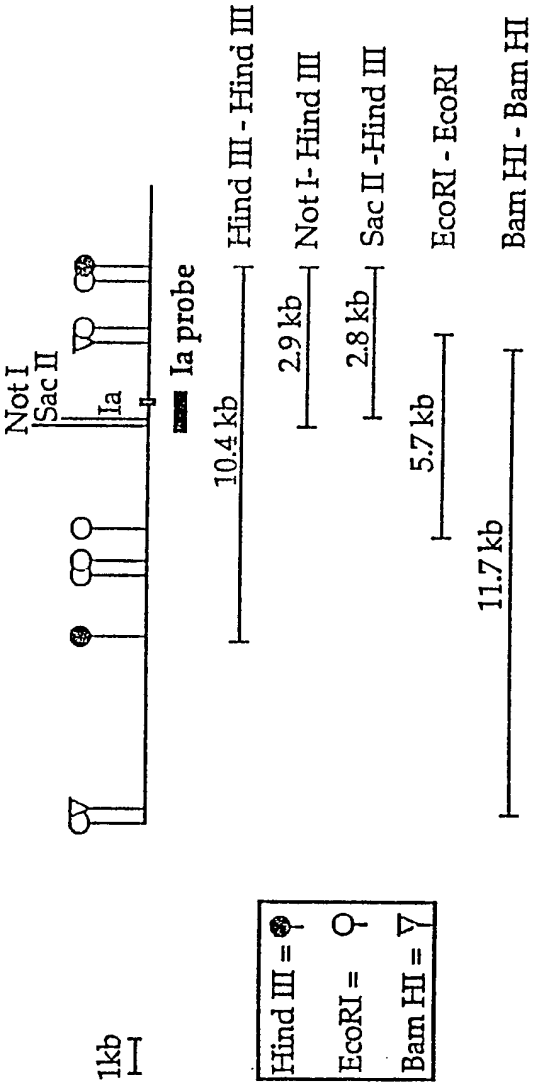
Not I
Sac II
Ia
Ia probe
10.4 kb
Hind III - Hind III
2.9 kb
Not I- Hind III
2.8 kb
Sac II -Hind III
5.7 kb
EcoRI - EcoRI
11.7 kb
Bam HI - Bam HI
1kb
Hind III =
EcoRI =
Bam HI =

Fig. 3A

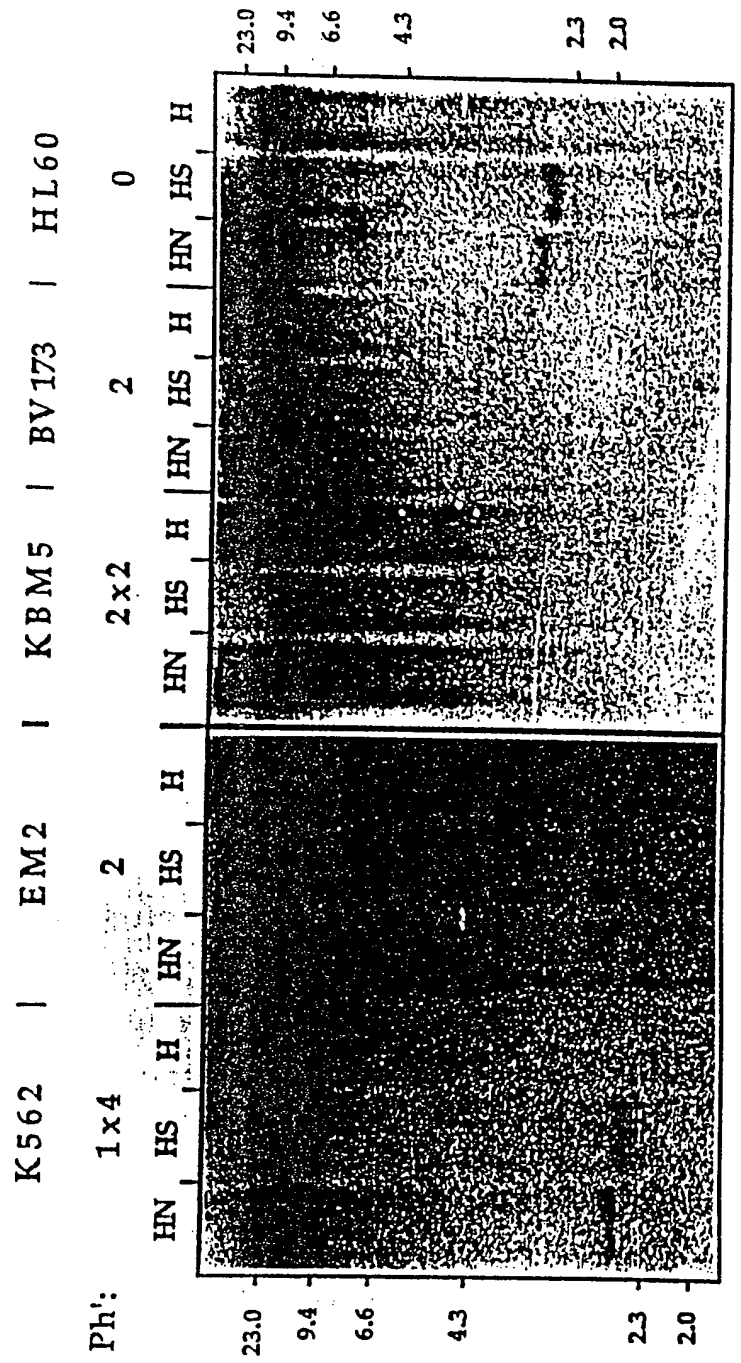
K562 | EM2 | KBM5 | BV173 | HL60
Ph':
1x4
2
2x2
2
0
HN HS H HN HS H HN HS H HN HS H HN HS H
23.0
9.4
6.6
4.3
2.3
2.0

Fig. 3B

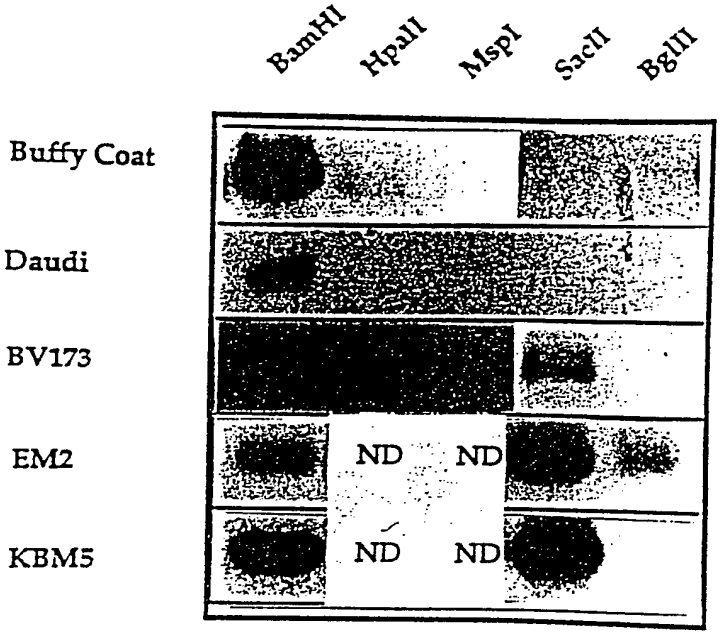
BamHI
HpaII
MspI
SacII
BglII
Buffy Coat
Daudi
BV173
EM2
KBM5
ND
ND
ND
ND

Fig. 3C

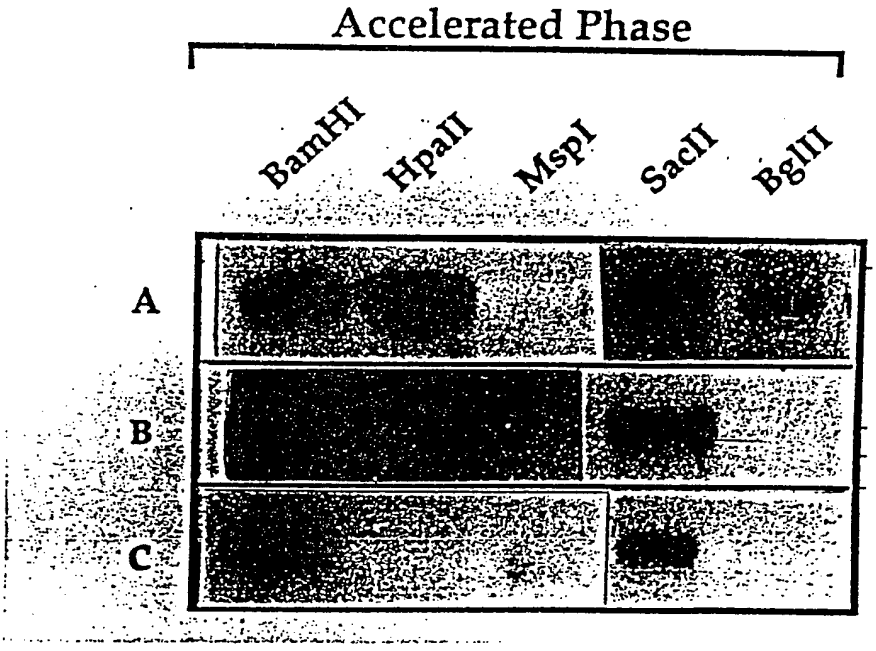

Fig. 4A

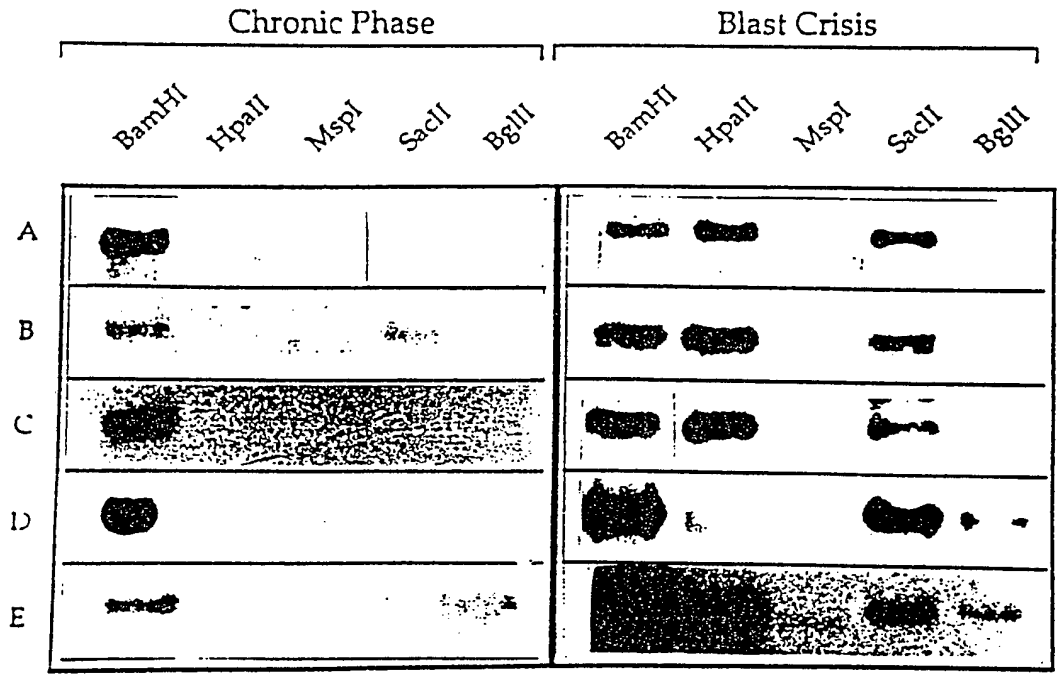
Chronic Phase
Blast Crisis
BamHI
HpaII
MspI
SacII
BglII
BamHI
HpaII
MspI
SacII
BglII
A
B
C
D
E

Fig. 4B

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 95/00966

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER DETECT PREV;9(1-2):9-15 1986, DEL SENNO L ET AL 'METHYLATION AND EXPRESSION OF C-MYC AND C-ABL ONCOGENES IN HUMAN LEUKEMIC K562 CELLS BEFORE AND AFTER TREATMENT WITogenD C-ABL ONCOGENES IN HUMAN LEUKEMIC K562 CELLS BEFORE AND AFTER TREATMENT WITH 5-AZACYTIDINE'	1-3
Y	see the whole document ---	4-10
X	CELL, vol. 47,no. 2, 1986 pages 277-284, E.SHTIVELMAN ET AL. 'Alternative splicing of RNAs transcribed from the human abl gen from the bcr-abl fused gene'	1-3
Y	see the whole document ---	4-10
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1995	28.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER BIOCHEM BIOPHYS;8(4):313-26 1986, TOBE H ET AL 'METHYLATION OF HUMAN HLA-D/DR GENES: DERANGEMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA'	1-3
Y	see the whole document	4-10
X	LEUKEMIA;7(5):707-11 1993, MILLS KI ET AL 'Methylation of the major breakpoint cluster region (M-bcr) in Philadelphia-positive CML.' see the whole document	1-3
X	LEUKEMIA;7(6):801-7 1993, OHYASHIKI JH ET AL 'The methylation status of the major breakpoint cluster region in human leukemia cells, including Philadelphia chromosome-positive cells, is linked to the lineage of hematopoietic cells.' see the whole document	1-3
A	US,A,5 057 410 (E.S.KAWASAKI ET AL.) 15 October 1991 see the whole document	4-10

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 95/00966

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5057410	15-10-91	NONE	